

Regulation of two photosynthetic pigment-related genes during stress-induced pigment formation in the green alga, *Dunaliella salina*

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Received: 23 September 2005 / Accepted: 8 February 2006
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Abstract The expression of mRNAs coding for 1-deoxyxylulose-5-phosphate synthase (DXS) and phytoene synthase (PSY) were studied in *Dunaliella salina* grown under nitrogen-sufficient (NS) and nitrogen-limited (NL) conditions. Under NS conditions growth was 2.5 times higher than under NL conditions. No differences were found in chlorophyll *a* content per cell, and total carotenoid content per cell was 5.33 pg 1⁻¹ for the NS treatment and 7.76 pg 1⁻¹ for the NL. DXS transcripts exhibited diminished expression under NL conditions, peaking at day 15 of cultivation in both treatments. Simultaneously, PSY transcripts exhibited constant expression under

both conditions. These results suggest that these genes play an important role in the balance of photosynthetic pigments during pigment accumulation.

Keywords Carotenoids · *Dunaliella salina* · Gene expression

Introduction

The halotolerant green alga *Dunaliella salina* is well known for its β -carotene production (Vorst et al. 1994). β -Carotene production in *D. salina* is enhanced by suboptimal growth conditions such as high light intensity, salinity or temperature (Vorst et al. 1994), or nutrient limitation (Marín et al. 1998). Commercial production of β -carotene using *D. salina* therefore must face the dilemma that β -carotene production is enhanced by physiological stress, even though this results in slower growth.

The mechanisms responsible for intracellular changes in pigment synthesis are not fully understood. Carotenoids are the final biosynthetic end-products of the isoprenoid synthesis pathway. The biosynthesis of isopentenyl diphosphate (IPP), the basic building block of isoprenoids, is catalyzed by 1-deoxyxylulose-5-phosphate synthase (DXS) coded by the *dxs* gene (Lichtenthaler 1999). Subsequent biosynthesis

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of carotenoids from IPP involves a common route including their conversion into geranylgeranyl diphosphate, which is the substrate for formation of phytol (chlorophyll side chain) and phytoene (Fig. 1). Phytoene formation is catalyzed by phytoene synthase (PSY) coded by the *psy* gene and is considered the first regulatory step in carotenogenesis, which culminates in β -carotene formation (Bartley and Scolnik 1995).

In the present study, *D. salina* was cultivated under nitrogen-sufficient and -limited conditions under moderate light levels to induce carotenogenesis. The aim was to examine the expression response of two genes involved in regulatory steps in carotenoid biosynthesis and monitor growth and pigment concentrations to better understand

the molecular basis of stress-induced pigment accumulation in the green alga *D. salina*.

Materials and methods

Dunaliella salina strain and culture conditions

D. salina (CDBB 500, CINVESTAV-IPN, Mexico) was cultivated in Johnson's medium (Johnson et al. 1968) at 20% NaCl (w/v) and under two nitrogen conditions: nitrogen-sufficient (1 g KNO₃ l⁻¹) and nitrogen-limited (Johnson's medium without KNO₃). Temperature was maintained at 30°C under a continuous irradiance of 300–350 $\mu\text{mol photon m}^{-2} \text{s}^{-1}$.

Measurement of cell growth and pigment content

Cells were counted with a hemacytometer (Cao et al. 2001). They were harvested by centrifuging at 3000g for 10 min and then fractionated. The resulting pellets were stored at -80°C until required.

Chlorophyll *a* was extracted from algal pellets in 10 ml methanol and supernatant absorbency was measured at 665 nm (Loeblich 1982). Total carotenoids were extracted from pellets in 5 ml acetone/water (80:20, v/v). The supernatant was measured at 480, 663 and 645 nm as described by Loeblich (1982).

Ribonuclease protection assay

Two hetero-riboprobes for cDNA catalytic and conserved domains in both the *psy* (698 bp, AF196963) and *dxs* genes (738 bp, AF479589) were obtained by using MAXIscript *in vitro* Transcription T7/T3 kits (Ambion, Austin, TX). Genes were obtained from annatto *Bixa orellana*, a plant with high carotenoid pigment content. A 158 bp ribosomal antisense riboprobe corresponding to the 28S subunit from *Camptotheca acuminata* cDNA (U42789) was used as control. Expression analysis was done using the ribonuclease protection assay (RPA) applied with the Direct Protect Kit (Ambion, Austin, TX).

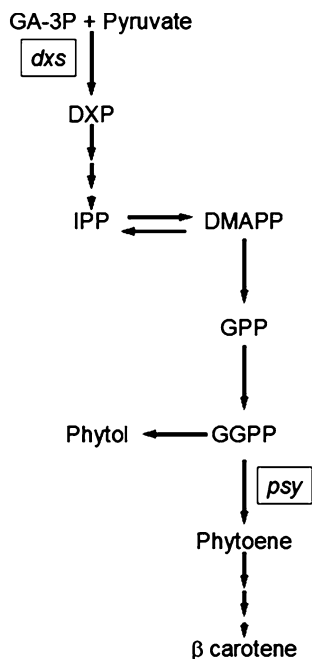


Fig. 1 Simplified pathway of photosynthetic pigment biosynthesis in algae. The *dxs* gene codes for 1-deoxyxylulose-5-phosphate synthase and the *psy* gene for phytoene synthase; both are shown inside the boxes. Abbreviations: GAP = glyceraldehydes 3-phosphate; DXP = 1-deoxyxylulose 5-phosphate; IPP = isopentenyl diphosphate; DMAPP = dimethylallyl diphosphate; GPP = geranyl diphosphate; FPP = farnesyl diphosphate; GGPP = geranylgeranyl diphosphate. Modified from Lichtenthaler (1999)

Results and discussion

Dunaliella salina cellular growth and pigment induction

D. salina grew better under NS conditions than NL conditions (Fig. 2). The difference in cell concentrations between treatments was significant beginning on day 10 of cultivation ($P < 0.05$). A similar effect on growth was observed in *D. salina* and *D. tertiolecta* under NL conditions (Marín et al. 1998).

Cells grown under NL conditions accumulated more pigment, showing a greater carotenoid content than the NS treatment cells. However, significant differences between treatments were only identified at day 20 (Table 1). This suggests that NL conditions favored faster pigment accumulation per cell, which became red-orange at the end of the culture period; similar results are reported by Marín et al. (1998).

Chlorophyll *a* content per cell exhibited similar behavior in both treatments (Table 1). No significant differences in total carotenoids to chlorophyll *a* ratios were observed between culture conditions, except on day 20 when a ratio of 2.65 was recorded. Although chlorophyll *a* level per cell remained constant, there is some evidence that nitrogen deficiency affects photosynthesis (Berges et al. 1996). Also, the photosynthetic apparatus is affected under irradiance stress, leading to chlorophyll photobleaching (Carpentier 1996) and a carotenogenic transformation of

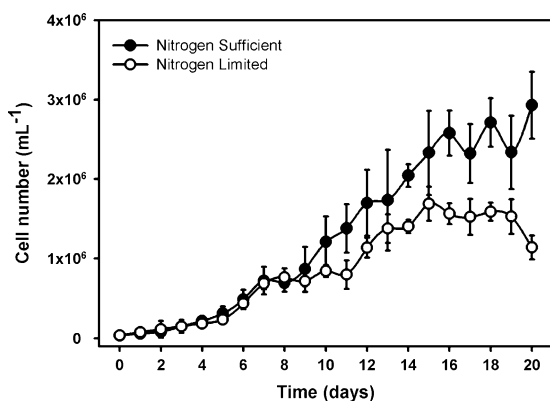


Fig. 2 Effect of nitrogen limitation on cellular growth in *D. salina*. Data are the means of three values \pm SE

Table 1 Photosynthetic pigment content per cell ($\mu\text{g l}^{-1}$) in *D. salina* cultures

Days	Chlorophyll <i>a</i>		Total Carotenoids	
	NL	NS	NL	NS
5	4.86 \pm 0.91	4.81 \pm 0.72	11.66 \pm 2.90	8.90 \pm 2.59
10	3.83 \pm 0.10	3.45 \pm 0.48	6.45 \pm 0.39	5.02 \pm 1.01
15	1.54 \pm 0.18	1.84 \pm 0.39	5.69 \pm 0.60	5.66 \pm 0.91
20	2.93 \pm 0.29	2.90 \pm 0.90	7.76 \pm 0.71	5.33 \pm 1.31

D. salina grown under nitrogen-sufficient (NS) and nitrogen-limited (NL) conditions

Results represent the mean value of experiments performed in triplicate \pm SD

chloroplasts to protect the photosynthetic system (Borowitzka and Borowitzka 1988), though they still possess thylakoids and perform photosynthetic reactions (Vorst et al. 1994).

Expression of regulatory genes under nitrogen-sufficient and -limited conditions

Transcriptional regulation of DXS mRNA was analyzed. Although DXS transcripts generally behaved similarly in both treatments, an increase in their expression under NS conditions was noted when compared to NL conditions (Fig. 3). The high DXS transcript levels found in cells grown under NS conditions may be due to the high growth rate experienced by these cells, which in turn leads to the high chloroplast development and photosynthetic activity levels needed to ensure active cellular growth (Loeblich 1982). Increases in DXS transcripts during plastid development have been thoroughly researched in plants (Lichtenthaler 1999).

Transcriptional activation of other genes related to isoprenoid precursor biosynthesis has been demonstrated in the microalga *Haematococcus pluvialis* (closely related to *D. salina*) where one of the genes coding for isopentenyl pyrophosphate isomerase was up-regulated in response to high light intensities (Sun et al. 1998). The present results show that under NL conditions the drop observed in DXS mRNA transcription was related to a constant chlorophyll *a* content per cell (Table 1). Alterations in plastid development during DXS transcript reduction have only been reported in an *Arabidopsis*

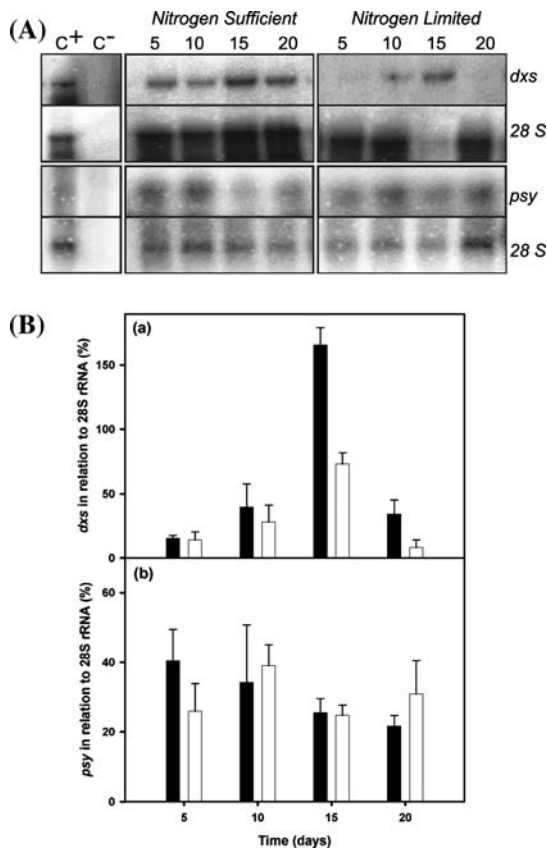


Fig. 3 Differential expression of DXS and PSY mRNA during culture period. **(A)** Representative RNAase-protected fragments resolved by 5% non-denaturing PAGE. **(B)** Estimate of mRNA steady-state levels of DXS (a) and PSY (b) mRNA done using fosforimager equipment (Bio Rad, Hercules, CA): Values are expressed as percentages of 28S rRNA signal. C-: RNAase activity control, C+: Positive control. Data are the means of two values \pm SE. Nitrogen-sufficient (-■-) and nitrogen-limited (-□-) culture conditions

thaliana cla-1 mutant, where DXS gene inactivation had a pleiotropic effect on plastid development and photosynthetic pigments accumulation (Mandel et al. 1996). Unlike the *Arabidopsis thaliana cla-1* mutant, however, *D. salina* grown under NL conditions increased its carotenoid content per cell at the end of the culture period (Table 1). This suggests that photosynthetic pigment formation is dependent on regulation of the carotenogenic enzymes located in the final steps of the isoprenoid synthesis pathway.

Analysis of PSY mRNA transcription showed that PSY mRNA expression was constant under

both culture conditions (Fig. 3). Similar results are reported in response to high irradiance stress (Rabbani et al. 1998). Unlike *D. salina*, however, *H. pluvialis* under stressful conditions reacts by transcribing this gene (Grünewald et al. 2000). Other carotenogenesis-related genes have been reported to up-regulate under stressful conditions in this alga (Steinbrenner and Linden 2001; Grünewald et al. 2000). Of note, however, is that under stressful conditions *H. pluvialis* accumulates carotenoids in the cytoplasm instead of the plastids, meaning accumulation of these metabolites in this organism does not affect plastid development (Sun et al. 1998).

In summary, the present results suggest that transcriptional regulation exists between the DXS and PSY transcripts in *D. salina* subjected to physiologic stress. Under NL conditions, DXS mRNA dropped in comparison to NS conditions. This decreased transcriptional effect is probably related to the drastic reduction in growth rate under NL conditions. In contrast, PSY mRNA, which is only involved in carotenoid production, was regularly transcribed, promoting carotenoid accumulation. This induced carotenogenesis was probably the result of a change in the photosynthetic pigment balance that favored carotenoid accumulation at the end of the experimental period.

Acknowledgments This research was financed by the CONACyT (J46435-Z) and SAGARPA (2002-C01-1057). The authors thank Margarita Aguilar Espinosa for technical assistance.

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